Impaired T-cell migration to the CNS under fingolimod and dimethyl fumarate

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ABSTRACT

Objective: To evaluate the long-term effects of treatments used in MS on the T-cell trafficking profile.

Methods: We enrolled 83 patients with MS under fingolimod (FTY), natalizumab (NTZ), dimethyl fumarate (DMF), or other disease-modifying treatments (DMTs). Blood was drawn before treatment onset and up to 36–48 months. The ex vivo expression of CNS-related integrins (α4β1 and α_L subunit of LFA-1) and the gut-related integrin (α4β7) was assessed using flow cytometry on $CD4^+$ and $CD8^+$ T cells. The adhesion profiles of $CD3^+$ T cells to specific integrin ligands (vascular cell adhesion molecule-1 [VCAM-1], intercellular adhesion molecule-1 [ICAM-1], and mucosal vascular addressin cell adhesion molecule-1 [MAdCAM-1]) were measured in vitro before and after 12 and 36–48 months.

Results: NTZ decreased the frequency of $\alpha4\beta1^+$ and $\alpha4\beta7^+$ integrin expressing T cells and the binding of these cells to VCAM-1 and MAdCAM-1, respectively. After 12 months, DMF induced a decreased frequency of $\alpha_L^{high}CD4^+$ T cells combined with reduced binding to ICAM-1. By contrast, with FTY, there was a doubling of the frequency of $\alpha4\beta1^+$ and α_L^{high}, but a decreased frequency of $\alpha4\beta7^+$ T cells. Strikingly, the binding of $\alpha4\beta1^+$, $\alpha4\beta7^+$, and to a lesser extent of α_L^{high} T cells to VCAM-1, MAdCAM-1, and ICAM-1, respectively, was decreased at month 12 under FTY treatment. The presence of manganese partially restored the binding of these T cells to VCAM-1 in vitro, suggesting that FTY interferes with integrin activation.

Conclusions: In addition to NTZ, DMF and FTY but not other tested DMTs may also decrease T-cell–mediated immune surveillance of the CNS. Whether this mechanism may contribute to the onset of CNS opportunistic infections remains to be shown. ***Neurol Neuroimmunol Neuroinflamm* 2017;4:e401; doi: 10.1212/NXI.0000000000000401**

GLOSSARY

ANOVA = analysis of variance; **DMF** = dimethyl fumarate; **DMT** = disease-modifying treatment; **FOV** = field of view; **FTY** = fingolimod; **GA** = glatiramer acetate; **HC** = healthy control; **ICAM-1** = intercellular adhesion molecule-1; **IFN** = interferon; **MACS** = magnetic-activated cell sorting; **MAdCAM-1** = mucosal vascular addressin cell adhesion molecule-1; **NTZ** = natalizumab; **PBS** = phosphate-buffered saline; **PBMC** = peripheral blood mononuclear cell; **PML** = progressive multifocal leukoencephalopathy; **RRMS** = relapsing-remitting MS; **VCAM-1** = vascular cell adhesion molecule-1.

MS is a debilitating disease resulting from combined genetic predisposition and environmental factors. Several agents have been approved for relapsing-remitting MS (RRMS), with varying efficacy and side-effect profiles. Fingolimod (FTY), dimethyl fumarate (DMF), and natalizumab (NTZ) are disease-modifying treatments (DMTs) used in MS, acting on immunoinflammatory components of the disease.[1] Although occurring more rarely than with NTZ, cases of progressive multifocal leukoencephalopathy (PML) have been reported in patients with MS treated with FTY and DMF. Moreover, other opportunistic CNS infections have been reported with FTY.[2–6] The occurrence of these CNS opportunistic infections raises the question whether these DMTs

Supplemental data at Neurology.org/nn

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may act on the immune surveillance of the CNS.[7] One hypothesis is that viral reactivation in the CNS may be related to a suboptimal immune surveillance via a modulation of the migratory profiles of T cells under FTY and DMF, such as it is observed with NTZ.

In this 4-year observational study, we aimed to better understand the long-term effects of DMF and FTY on the maintenance of T-cell–mediated CNS immune surveillance. We compared those 2 drugs with NTZ, known to block the function of α4β1 and α4β7 integrins involved in CNS and gut T-cell homing, respectively, and to other DMTs (interferon [IFN]-β, glatiramer acetate [GA], teriflunomide), which have not been associated with CNS infections. Specifically, we examined the effect of these different DMTs on the expression and function of integrins previously shown to mediate T-cell homing to the CNS or the gut by comparison with NTZ mechanism of action.

METHODS **Subjects.** We enrolled 83 patients with RRMS treated with (1) FTY (0.5 mg/d, n = 38), (2) NTZ (300 mg iv monthly, n = 14), (3) DMF (2 × 240 mg/d, n = 20), or (4) "other DMTs" (IFN-β 1a [3 × 44 μg/wk sc or 30 μg/wk im], n = 8, GA [20 mg/d sc]: n = 2, teriflunomide [14 mg/d]: n = 1) and followed them up to 48 months into treatment (table). Fourteen healthy controls (HCs) were also enrolled. The diagnosis of MS was made using the revised McDonald criteria.[8] All blood samplings were performed at least 3 months after corticosteroid therapy. Blood samples were drawn just before (T0) and at 6, 12, 24, and 36–48 months into MS treatment or until its interruption (table). For NTZ, blood draw was performed just before the

Table Clinical data of patients enrolled

	Age at study entry,[a] y	F:M ratio	Disease duration,[a] y	EDSS score[a]	Total no. of relapses before treatment onset[a]	Total no. of relapses during the period of the treatment[a]	Previous treatment[b]	No. of patients reaching time point
FTY cohort (n = 38)[c]	39/14	26:12	9/9	1.5/0.5	3/4.5	0.0/0.0	16 IFN-β 1a	T6: 38
							3 IFN-β 1b	T12: 38
							6 GA	T24: 36
							3 NTZ	T36-48: 29[d]
							2 cladribine (phase III trial)	
							8 without	
NTZ cohort (n = 14)	37.5/12	13:1	5.5/7	2.75/2	3/4	0.0/1.0	5 IFN-β 1a	T6: 6
							3 IFN-β 1b	T12: 14
							4 GA	T24: 12
							1 mitoxantrone	
							1 FTY	
DMF cohort (n = 20)	42.5/16.5	13:7	9/11	1.5/1	2/3	0.0/0.0	6 IFN-β 1a	T6: 11
							2 IFN-β 1b	T12: 20
							1 GA	T24: 0
							1 teriflunomide	
							10 without	
Other DMT cohort[e] (n = 11)	37.5/13	9:2	1.5/11	1.5/1	2/2.5	0.0/0.0	2 IFN-β 1a	T6: 10
							1 GA	T12: 9
							8 without	T24: 4
HC cohort (n = 14)	29.9/9.1	5:9	NA	NA	NA	NA	NA	NA

Abbreviations: DMF = dimethyl fumarate; DMT = disease-modifying treatment; EDSS = Expanded Disability Status Scale; FTY = fingolimod; GA = glatiramer acetate; HC = healthy control; IFN-β = interferon-beta; NA = not applicable; NTZ = natalizumab.

[a] Numbers represent the median/interquartile range.

[b] Washout period in between treatments in months (median/interquartile range): FTY cohort = 0.0/1.3; NTZ cohort = 0.3/1.7; DMF cohort = 0.1/0.0; other DMT cohort = 13.8/11.0.

[c] Twenty-nine patients were tested for phenotypes and functional assays presented in figures 1–3; and 9 for functional assays presented in figure 4.

[d] Ten patients reaching T36 and 19, T48.

[e] Eight patients under IFN-β 1a; 2 under GA, and 1 under teriflunomide.

next injection (e.g., blood sample at T6 was drawn just before the seventh cure). Peripheral blood mononuclear cells (PBMCs) were isolated and frozen as previously described within 4 hours after blood draw.[9]

Standard protocol approvals, registrations, and patient consents. Enrolled patients were part of an ongoing open study aiming at characterizing the effects of disease-modifying therapies. This study was accepted by our institution's review board (protocol 107/13), and all participants provided their written informed consent.

Ex vivo surface staining for flow cytometry analyses. To assess the ex vivo level of expression of different surface markers, thawed PBMCs[9,10] were stained at 4°C for 30 minutes with the following antibodies: CD3-APC-Cy7, CD8-Pacific Blue (both from Becton Dickinson, Franklin Lakes, NJ), CD4-ECD (Beckman Coulter, Brea, CA), CD11a-FITC (α_L), CD49d-APC (α4 integrin, both from Becton Dickinson), β7 integrin PE (eBioscience, San Diego, CA), and CD29-Alexa700 (β1 integrin; BioLegend, San Diego, CA).[11] Dead cells were excluded using the Aqua LIVE/DEAD stain kit (Invitrogen AG, Waltham, MA). A blinded investigator acquired the data on an LSRII flow cytometer (Becton Dickinson) and analyzed using FlowJo software (version 8.8.6; Tree Star Inc., Ashland, OR). All samples from a given study participant were analyzed on the same day; each set of experiments included patients from 2 to 3 different categories of DMTs and at least 1 HC so that potential sources of variation and technical side effects were minimized. All experiments were performed within a short period of time, using the same lots of antibodies and the same cytometer settings. Ex vivo analyses were performed on T cells expressing high levels of α_L integrin subunit (α_L^{high}) or T cells coexpressing either α4 and β1 integrin subunits ($\alpha4\beta1^+$ T cells) or the α4 and β7 integrin subunits ($\alpha4\beta7^+$ T cells) (see figure e-1 at Neurology.org/nn for detailed gating strategy).

$CD3^+$ T-cell adhesion to integrin ligand-coated slides. For functional binding assays, $CD3^+$ T cells were sorted within thawed PBMCs by magnetic-activated cell sorting (MACS) using anti-CD3 MicroBeads with an autoMACS Pro Separator (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The purity of sorted cells was checked by flow cytometry using CD3-APC-Cy7 antibodies and counterstaining with Aqua LIVE/DEAD. In vitro binding assays were performed only if the purity of sorted populations reached at least 90%. Binding profiles of isolated $CD3^+$ T cells were assessed as described.[12] Briefly, recombinant purified cell adhesion molecules (human intercellular adhesion molecule-1 [ICAM-1]/Fc, vascular cell adhesion molecule-1 [VCAM-1]/Fc, and mucosal vascular addressin cell adhesion molecule-1 [MAdCAM-1]/Fc or control Delta/Notch-like EGF-related receptor/Fc [100 nM; from R&D Systems, Minneapolis, MN]) were bound to epoxy slides (Thermo Scientific, Waltham, MA), precoated with protein A (BioVision, Milpitas, CA), and blocked with 1.5% bovine serum albumin in phosphate-buffered saline (PBS) (pH 7.4) for 30 minutes before use. One hundred thousand $CD3^+$ T cells/field were then incubated at room temperature for 30 minutes on a rotating platform. To measure the maximal level of integrin-mediated adhesion, $CD3^+$ T-cell integrin affinity maturation was induced by incubation of T cells in the presence of 1 mM $MnCl_2$ (Sigma Aldrich, St. Louis, MO) for 30 minutes. After washing steps in PBS, adhering T cells were fixed with 2.5% (vol/vol) glutaraldehyde in PBS for at least 2 hours on ice. All samples from a given study participant were analyzed on the same day; each set of experiments included patients from 2 to 3 different categories of DMTs and at least 1 HC so that potential sources of variation and technical side effects were minimized. The number of cells per field of view (FOV) was quantified in a blinded fashion using a counting ocular with a classical optical microscope (Zeiss AxioSkop 40 ×20, objective Carl Zeiss 1016-758, W-pi ×10).

Statistical analysis. Statistical analysis was performed with GraphPad Prism software, version 7.00. To take into account the few missing value, a Kruskal-Wallis analysis of variance (ANOVA) was run to test (1) differences in between the 3 groups of treatments only at T0 (FTY vs NTZ vs DMF vs other DMTs) and (2) the effect of each treatment all along the treatment duration (FTY, NTZ, DMF, or the other DMTs, T0 vs T6 vs T12 vs T24 vs T36–48). p Values obtained from these ANOVA are mentioned in the text as p_{KW}. If the effect of the treatment reached significance, the differences between immune responses measured at T0 with the various subsequent time points were analyzed with the nonparametric paired Wilcoxon ranked test. Unless mentioned otherwise in the figure legend, a Bonferroni correction was performed to take into account the comparisons performed at multiple time points (T0 vs T6, T0 vs T12, T0 vs T24, and T0 vs T36–48). $p < 0.05$ was considered statistically significant for adjusted p values.

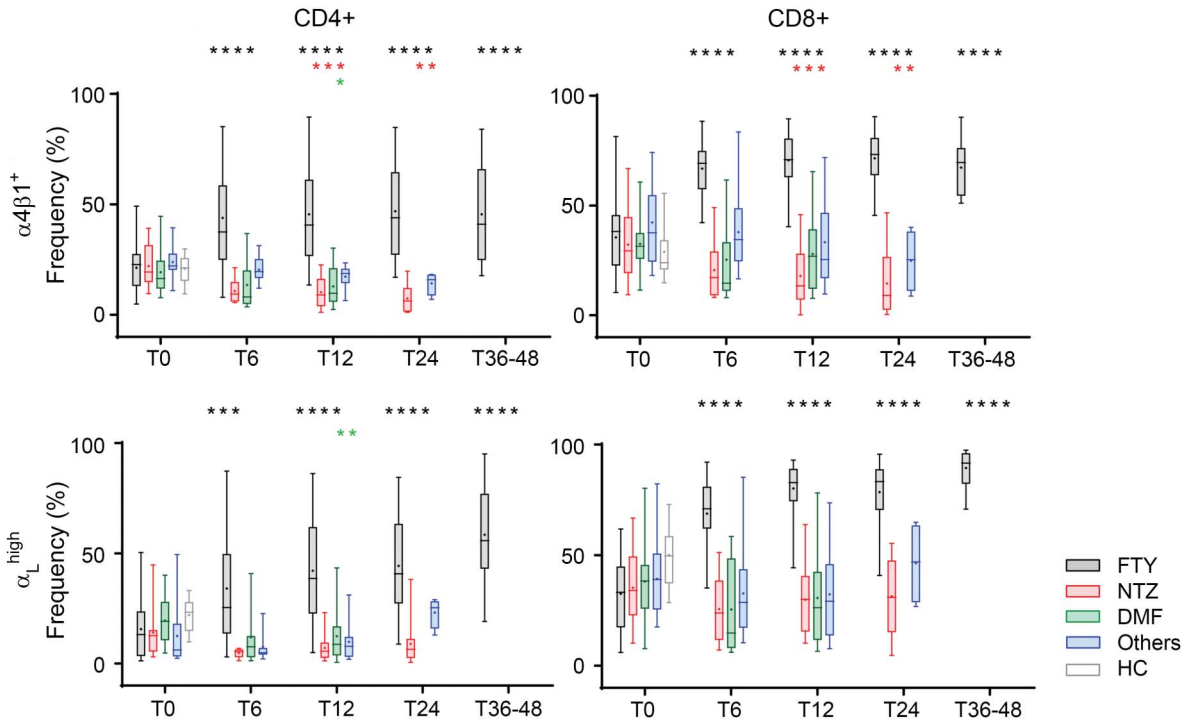
RESULTS

FTY and DMF treatments affect T-cell homing to the CNS and the gut. ***Integrins involved in T-cell homing to the CNS.*** We first assessed changes in the ex vivo profile of T-cell homing phenotypes characteristic for the CNS and the gut under the 4 groups of DMTs by determining the surface expression of integrins on $CD4^+$ and $CD8^+$ T cells using flow cytometry. Those were (1) the CNS-addressing integrin $\alpha4\beta1$ and the integrin subunit α-chain of LFA-1 ($\alpha4\beta1^+$, α_L^{high}, figure 1)[13–17] and (2) the gut-addressing integrin: α4β7 ($\alpha4\beta7^+$, figure 2).[18,19] No differences in between the 5 groups of participants (patients with MS and HCs) could be detected before treatment onset, i.e., at T0 as tested by Kruskal-Wallis tests.

In the group of other DMTs, none of the markers studied were associated with fluctuations in integrin expression on T cells during the entire treatment duration (figures 1 and 2, data in blue). As expected, our findings confirmed that, under NTZ, the frequency of $\alpha4\beta1^+$ integrin–expressing $CD4^+$ and $CD8^+$ T cells, especially at T12 and T24 (figure 1, data in red) was reduced,[11,20,21] supporting the notion that T-cell trafficking to the CNS is diminished. We also observed, at T24, a 2.2- and a 1.5-fold decrease in the frequency of expression of the α_L subunit of LFA-1 on $CD4^+$ and $CD8^+$ T cells, respectively, however without reaching significance (CD4: $p_{KW} = 0.057$; CD8: $p_{KW} = 0.670$).

The situation was completely different in the case of FTY. As expected, there was a global reduction of both, $CD4^+$ and $CD8^+$ T-cell counts in the peripheral blood (data not shown). However, there was a highly significant increase in the frequency of

Figure 1 The CNS-trafficking phenotype of T cells is affected by NTZ, FTY, and DMF



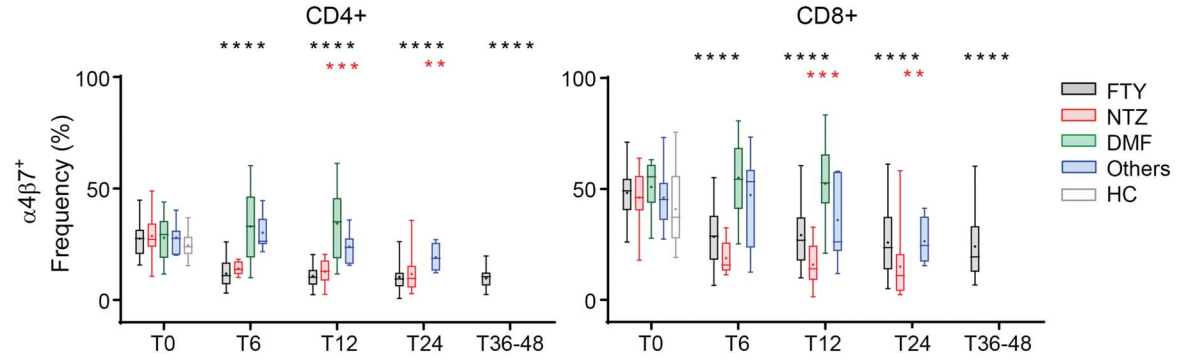
Frequencies of $\alpha4\beta1^+$ or α_L^{high} T cells were assessed by flow cytometry in $CD3^+CD4^+$ (left panels) or $CD3^+CD8^+$ T cells (right panels). Data were obtained before treatment initiation (T0) and after 6 (T6), 12 (T12), 24 months (T24), and 36–48 months (T36–48) of treatment. Data of FTY-treated patients with MS appear in black in the graphs (n = 29), NTZ in red (n = 14), DMF in green (n = 20), patients under other DMTs in blue (n = 11), and HCs in gray (n = 14). Box and whiskers plots show median, 25th and 75th percentiles, and range. The mean is represented as a dot in the box. The asterisks represent significant differences for the effect of time for a given treatment: in black for FTY-treated patients; in red, NTZ-treated patients; and in green, DMF-treated patients. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. DMF = dimethyl fumarate; DMT = disease-modifying treatment; FTY = fingolimod; HC = healthy control; NTZ = natalizumab; Others = other DMTs.

circulating T cells expressing CNS-related integrins ($\alpha4\beta1^+$ or α_L of LFA-1) at all time points tested. The median frequency of $\alpha4\beta1^+$-expressing $CD4^+$ and $CD8^+$ T cells, as measured at T6, T12, T24, and T36–48, exceeded baseline median values by 65%–92% ($p_{KW} < 0.0001$) and 81%–92% ($p_{KW} < 0.0001$), respectively (figure 1, data in black). The enhanced frequency of α_L-expressing $CD4^+$ and $CD8^+$ T cells was even more impressive, being 1.9–4.2-fold ($p_{KW} < 0.0001$) and 2.1–2.7-fold ($p_{KW} < 0.0001$) increased, respectively.

On DMF, a reduction of 35% and 36% in the frequency of $CD4^+$ T cells expressing $\alpha4\beta1$ or α_L, respectively, was present at T12, while no effect was noted within $CD8^+$ T cells (figure 1, data in green).

Integrins involved in T-cell homing to the gut. Of interest, in the cohort of FTY-treated patients, the expression profile of $\alpha4\beta7$ integrin–mediating T-cell

Figure 2 The gut-trafficking phenotype of T cells is downregulated by NTZ and FTY



The frequency of $\alpha4\beta7^+$ T cells was assessed by flow cytometry in $CD3^+CD4^+$ (left panels) or $CD3^+CD8^+$ T cells (right panels). The color code, the number of patients tested, the statistics, and the abbreviations are exactly the same as in figure 1 (see legend to figure 1).

homing to the gut was completely different from that observed for α4β1 integrin and the subunit α_L of LFA-1. Indeed, we found that the frequency of α4β7$^+$-circulating CD4$^+$ and CD8$^+$ T cells decreased at least by 65% and 42%, respectively. This pattern was similar to what is observed for NTZ, which targets both α4β1 and α4β7 integrin–expressing T cells (figure 2). By contrast, there was no significant change in the frequency of α4β7$^+$ integrin–expressing circulating T cells on DMF or other DMTs.

T-cell adhesion profiles under DMTs used in MS. Cell surface coexpression of integrin subunits does not suffice to determine proper integrin function that requires a functional heterodimer and additional activation by integrin affinity maturation. Thus, in a next step, we determined whether MS DMTs have an effect on integrin-mediated binding to their respective endothelial ligands. To this end, we performed in vitro binding assays focusing on the ability of CD3$^+$ T cells to bind to VCAM-1 and ICAM-1 involved in T-cell homing to the CNS and MAdCAM-1 involved in T-cell homing to the gut (figure 3).

First, we did not observe any significant difference in the adhesion of circulating T cells to all adhesion molecules tested in the cohort of other DMTs (figure 3, data in blue). Regarding NTZ, as expected, we found that the number of CD3$^+$ T cells adhering to VCAM-1 and MAdCAM-1 dropped significantly from 65 to 22 and from 7 to 2 cells/FOV, respectively (figure 3, data in red). In line with the ex vivo phenotype (figures 1 and 2), no change in the binding profile of T cells to ICAM-1 under NTZ was noted ($p = 0.375$; figure 3B, data in red). By contrast, DMF induced a significant decrease in the median number of ICAM-1–adhering T cells/FOV at T12, dropping from 61 to 40 cells ($p = 0.039$; figure 3B, data in green). However, with DMF, there was no decreased adhesion to VCAM-1 or MAdCAM-1.

We finally demonstrated that FTY treatment interferes with the interaction between CNS- and gut-related integrins (α4β1; α4β7) and their respective ligands (VCAM-1 and MAdCAM-1) (figure 3). Here, we observed a drop in CD3$^+$ T-cell adhesion to VCAM-1 ($p = 0.027$) and to MAdCAM-1 ($p = 0.0078$) but not to ICAM-1 ($p = 0.570$), after 1 year of FTY treatment. This functional profile strikingly resembles what was detected in NTZ-treated MS patients. Of note, this initial decrease in binding to integrin ligands under FTY is followed by a rescue of the profile of adhesion of T cells to VCAM-1 and MAdCAM-1 or even, in the case of ICAM-1, an increased adhesion profile, as observed at T36–48 (figure 3, data in black). Thus, although the circulating pool of T cells shows an increased frequency of cells expressing α4β1 and αL, FTY seems to cause a transient reduced ability of these T cells to bind to the integrin ligands VCAM-1 and ICAM-1.

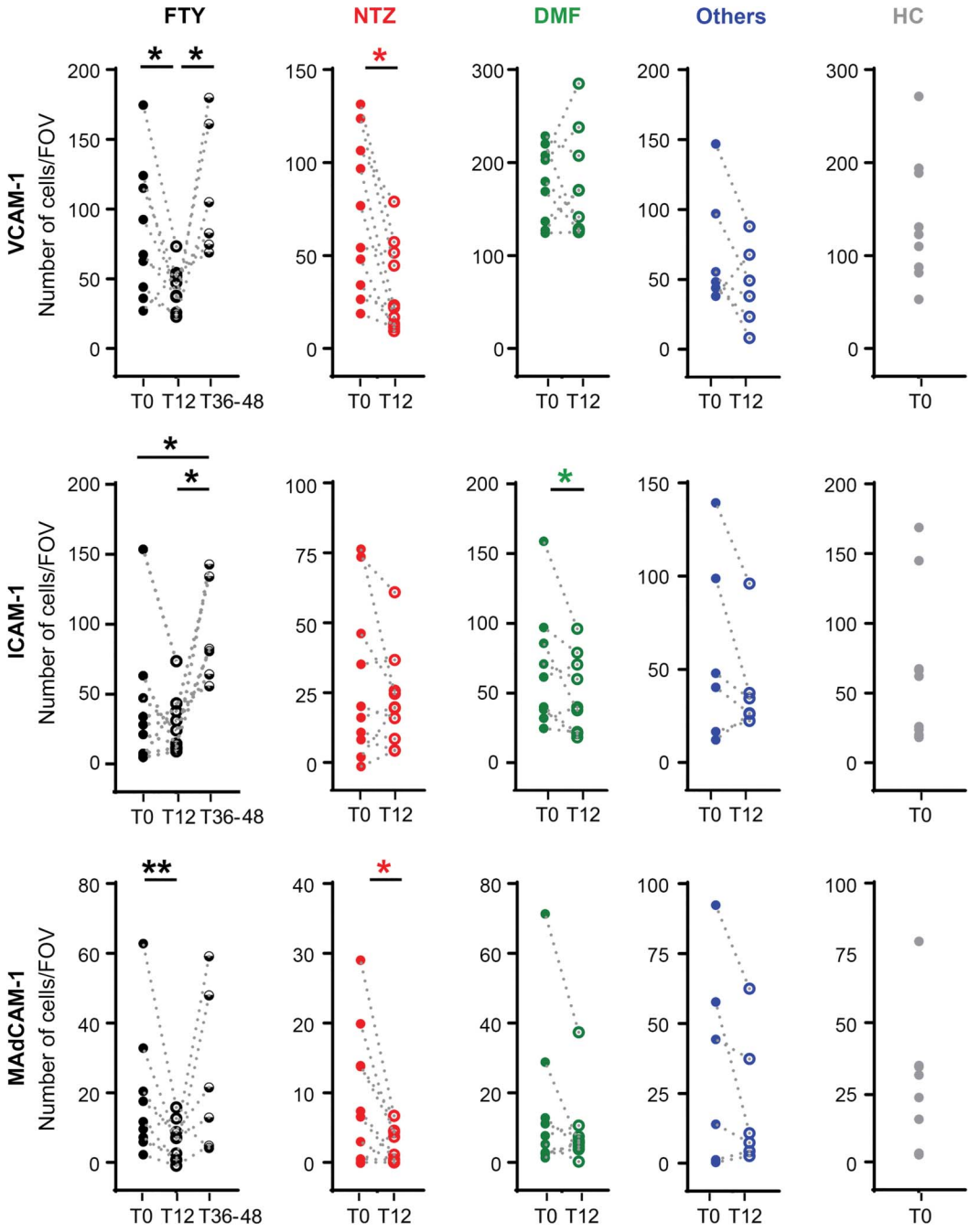
To investigate whether T-cell ability to adhere to VCAM-1 in FTY-treated patient samples could be chemically restored in vitro, binding assays in the presence of $MnCl_2$, which induces integrin affinity maturation, were performed.[22] Of interest, the addition of $MnCl_2$ partially restored binding to VCAM-1 ($p_{KW} = 0.057$) even if the median number of cells/FOV was still lower at T12 (121) as compared to T0 (183) (figure 4, +$MnCl_2$). These results further suggest that FTY, beside a strong lymphopenic activity, would also interfere, in an at least-partially reversible way, with the activation profile of α4β1 integrins expressed on the surface of T cells, further impeding their access to the CNS.

DISCUSSION Several effective treatments are available for patients with RRMS, but some of them are associated with severe side effects. NTZ has a strong efficacy but is associated with a consistent risk of developing PML (711 cases as of March 6, 2017).[23] NTZ is a humanized monoclonal IgG4 antibody targeting the α4 integrin subunit of α4β1, preventing the entry of autoreactive T cells into the CNS, but probably also of CD8$^+$ T cells fighting viral infections, thus decreasing the immune surveillance and favoring the occurrence of PML.[24,25] Intriguingly, cases of PML have also been reported with 2 other DMTs, namely FTY (13 cases in patients who had not been previously on NTZ, as of July 13, 2017) and DMF (5 cases in patients with MS and 11 in patients with psoriasis treated with DMF-related products). Here, we examined whether an impairment of the immune surveillance could also be involved with these 2 DMTs and thus be a contributing factor to the occurrence of opportunistic infections.

First, we were able to confirm that NTZ reduces the frequency of integrin-expressing circulating T cells either directed to the CNS (α4β1$^+$ and α_L^{high}, figure 1)[11,20,21] or to the gut (α4β7$^+$, figure 2). Furthermore, NTZ diminished the level of expression of these integrins on T cells, as detected by decreased mean fluorescence intensities (MFI) (data not shown). Decreased detection of α4β1 or α4β7 may be due to epitope masking by NTZ or alternatively by NTZ-induced integrin internalization.[26] This decreased expression of integrins was associated with a reduced adhesion of T cells to both, VCAM-1 and MAdCAM-1, confirming that NTZ is highly efficient to decrease the migration of T cells into the CNS or into the gut.

The mode of action of phosphorylated FTY consists in the internalization of sphingosine-1-phosphate (S1P) receptors expressed by T cells. This internalization prevents the egress of naive (T_N) and

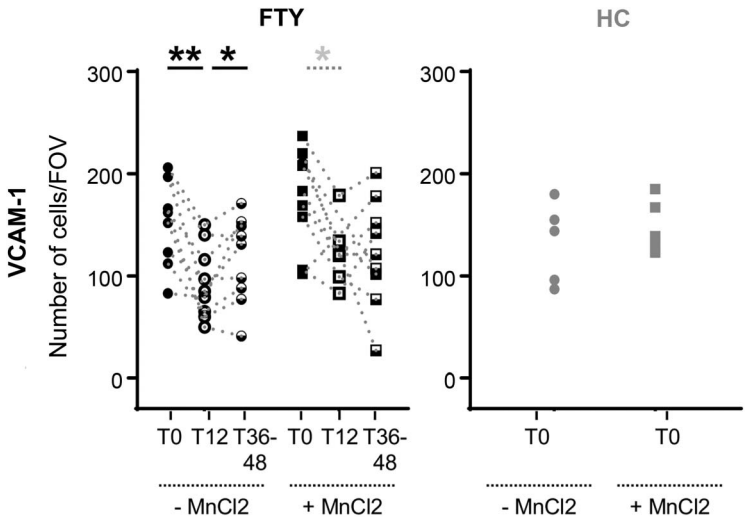
Figure 3 The binding of integrins to their ligands is affected by NTZ, FTY, and DMF



The numbers of CD3+ T cells adhering to VCAM-1, ICAM-1, and MAdCAM-1–immobilized receptors were quantified in an in vitro binding assay. Data were obtained before treatment initiation (T0) and after 12 months (T12) and 36–48 months (T36–48) of treatment. Background values obtained from the control DNER receptor were subtracted from the number of cells adhering to VCAM-1, ICAM-1, or MAdCAM-1. Each dot represents the mean value of 9 individual counts (3 counts per triplicates). Data of FTY-treated patients with MS appear in black (n = 9), NTZ in red (n = 10), DMF in green (n = 9), patients under other DMTs in blue (n = 6), and HCs in gray (n = 9). The statistical method is the same as in figures 1 and 2 (see legend to figure 1). *$p < 0.05$; **$p < 0.01$. DMF = dimethyl fumarate; DMT = disease-modifying treatment; DNER = Delta/Notch-like EGF-related receptor; FOV = field of view; FTY = fingolimod; HC = healthy control; ICAM-1 = intercellular adhesion molecule-1; MAdCAM-1 = mucosal vascular addressin cell adhesion molecule-1; NTZ = natalizumab; Others = other DMTs; VCAM-1 = vascular cell adhesion molecule-1.

central memory (T_{CM}) cells expressing the chemokine receptor CCR7 from lymph nodes into the blood stream.[27] This is of particular interest since peripheral T_{CM} cells are thought to be the efferent limb of CNS autoimmunity.[28] By contrast, effector memory (T_{EM}) and effector (T_E) T cells, which do not express CCR7, can exit from the lymph node, even in the presence of FTY. Thus, the immune surveillance of the CNS, performed by T_{EM} would not be affected. In the present study, we extend our understanding of the mechanisms of FTY. Indeed, we show that under this drug, there is an increased frequency of T cells in the blood

Figure 4 VCAM-1–binding profile of T cells isolated from FTY-treated patients is partially restored on manganese treatment



The number of cells adhering to VCAM-1 was assessed in vitro among magnetic-activated cell sorting–isolated $CD3^+$ T cells treated or not with $MnCl_2$. Background values obtained from the control DNER receptor were subtracted from the number of cells adhering to VCAM-1. Each dot represents the mean value of 9 individual counts (3 counts per triplicates). Data of FTY-treated patients with MS appear in black (n = 9) and HC in gray (n = 5). The effect of the treatment duration for the FTY-treated group was assessed using Kruskal-Wallis tests ($p = 0.019$ in the absence of $MnCl_2$ and $p = 0.054$ in the presence of $MnCl_2$). The effect of the FTY treatment was finally tested using pairwise nonparametric Wilcoxon tests. Asterisks represent statistically significant differences for the effect of time for a given treatment: in black if the Kruskal-Wallis test was statistically significant; in gray if not. $*p < 0.05$; $**p < 0.01$. DNER = Delta/Notch-like EGF-related receptor; FOV = field of view; FTY = fingolimod; HC = healthy control; VCAM-1 = vascular cell adhesion molecule-1.

expressing integrins involved in T-cell homing to the CNS ($\alpha4\beta1^+$ and α_L^{high} of LFA-1; figure 1), but a decreased frequency of those involved in T-cell homing to the gut ($\alpha4\beta7^+$; figure 2). This modification in terms of frequency is associated with a slight increase in the MFI of the $\alpha4$ and α_L subunits, but not of the $\beta1$ nor $\beta7$ expression (data not shown). The increased frequency of CNS integrin–expressing T cells in patients on FTY is explained by the higher proportion of CCR7-negative circulating T cells (T_{EM} and T_E), which, precisely, express more $\alpha4\beta1$ and α_L of LFA-1, but less $\alpha4\beta7$ than CCR7+ T cells, i.e., T_N and T_{CM} (data not shown). More surprising, we report that these $\alpha4\beta1^+$-expressing T cells exhibit a transitory, but important, decrease in their binding to VCAM-1 at T12 (figure 3), suggesting that FTY impairs affinity maturation and thus function of $\alpha4\beta1$ integrin. Of interest, the addition of manganese partially restores integrin affinity maturation as demonstrated by the partial induction of T-cell binding to the $\alpha4\beta1$ integrin ligand VCAM-1 (figure 4), suggesting that FTY, at least ex vivo, indeed may induce a suboptimal function of integrins. Such as for $\alpha4\beta1$, there is an increased binding of αL-expressing T cells to ICAM-1 after 3 years of treatment. Finally, similar to the $\alpha4\beta1$ integrin/VCAM-1 couple, the binding of $\alpha4\beta7$ to MadCAM-1 is transitorily, but markedly, reduced after 1 year of treatment. Although this remains hypothetical, it is possible that the decreased $\alpha4\beta1$ integrin–mediated binding of circulating $CD3^+$ T cells to VCAM-1 may play a contributing role in the CNS opportunistic infections that are observed under FTY treatment.

The picture is different in the case of DMF. We demonstrate that this DMT has a strong effect on the expression and function of α_L, the integrin subunit of LFA-1, but not of $\alpha4\beta1$ or $\alpha4\beta7$. Indeed, DMF induces a 46% reduction in the frequency of circulating αL-expressing T cells, and, by doing so, decreases by 36% the number of T cells adhering to ICAM-1. These results may bring some light on the mode of action of DMF. The classic view poses that DMF promotes the antioxidative stress cell machinery working as a transcriptional promoter downstream to the activation of the transcription nuclear factor erythroid-2-related factor 2 (Nrf2).[29] But DMF has also been shown to redirect cytokine production toward a Th2 and M2 profile on lymphocytes and microglial cells, respectively.[30,31] Furthermore and of interest, based on in vitro models, others have suggested that DMF could inhibit cytokine-induced E-selectin, VCAM-1, and ICAM-1 expressions in human endothelial cells. DMF would thus potentially disturb T-cell migration to the CNS.[32,33] Our data showing a strong inhibitory effect of DMF on LFA-1–mediated binding of circulating T cells to ICAM-1 support this hypothesis,

suggesting that preventing activated T cells to cross the blood brain barrier may be a significant mode of action of DMF. A blood lymphocyte count below 500/mm^3 has been observed in DMF-induced PML cases.[34,35] CD8$^+$ T cells are disproportionally reduced as compared to CD4$^+$ T cells,[36] a factor that may favor the onset of PML.[37,38] Of note, DMF-related PML cases also occurred in patients with a blood lymphocyte count above 500/mm^3.[39,40] Our data suggest that DMF, by decreasing the LFA-1 binding of circulating T cells to ICAM-1, may impede the immune surveillance of the CNS, which may contribute to explain the occurrence of PML in DMF-treated patients, in particular those who do not present with lymphopenia.

Contrasting with the 3 other DMTs, we do not observe significant changes in the expression or the function of integrins on the circulating T-cell pool in the group of other DMTs. And precisely, IFN-β, GA, or teriflunomide has not been associated with CNS opportunistic infections, in particular PML. Nevertheless, we acknowledge the fact that the category of other DMTs encompasses fewer patients than the 3 other groups of DMTs. Furthermore, the other DMTs category is composed of 3 different compounds, IFN-β being by far the most represented. Yet, supporting the validity of our data, there is not even a trend for a decreased frequency of integrin-expressing circulating T cells or a diminished adhesion of T cells to endothelial ligands in the other DMTs group. It can also be noted that the values are quite close to the median (figures 1 and 2), suggesting that this group is relatively homogenous. Thus, we conclude that the data of the other DMTs group can be taken into consideration, despite the relatively small sample size.

To conclude, we report for the first time, that not only NTZ but also FTY and DMF may, by different mechanisms, impair the T-cell–mediated immune surveillance of the brain. These results, if confirmed by others, may open new axes of research to explain the few cases of PML associated with these drugs.

AUTHOR CONTRIBUTIONS

A.M. designed research, performed the experiments, analyzed data, and wrote the manuscript. S.P. discussed the results and revised the manuscript. M.C., C.B., and C.G. performed the experiments and analyzed data. B.E. contributed to research design, discussed the results, and revised the manuscript. M.S. enrolled patients, provided clinical data, and revised the manuscript. R.D.P. designed research, wrote the manuscript, and supervised the study.

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